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Equilibrium shift by target DNA substrates for determination of DNA binding ligands

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Abstract—An equilibrium containing the thiol derivative of Hoechst33258 (Ht-SH), glutathione (G-SH), and the corresponding homo and hetero disulfides was shifted by the addition of the duplex DNA. It was shown from the analysis of the components that the hetero disulfide Ht-SS-G increased by binding with the DNA (CA14) with an A_3T_3 binding motif for the structure of Hoechst33258, and that the different equilibrium shift was observed in the presence of CT14 with no A_3T_3 binding motif.

Higher-order structures of DNA and RNA play significant roles in regulation of gene expression,[1] and development of small molecular ligands for targeting such structures has become of great interest. There are a number of ligands varying from naturally occurring compounds to the designed ones for targeting non-canonical nucleic acids such as nucleosomal DNA,[2] quadruplex,[3] triplet repeats,[4] branched DNA,[5] and so on. Nevertheless, design of specific ligands for predetermined biological targets is difficult, hampering prompt development of effective ligands for higher-order nucleic acids. In an approach to recognition molecules for non-canonical DNA structure with small molecules, we have recently reported the strategy by using derivatives of minor-groove binders, in which assembly of the known DNA binders should produce different binding specificity.[6,7] The ligands connecting two Hoechst33258 units (Bis-Hoechst) exhibited selective binding toward the DNA sequence with two remote A_3T_3 binding sites as well as for the three-way junction DNA (Fig. 1A).[6] The structure of the new Hoechst ligand is characterized by the polyether-linker that is introduced at the convex side of the Hoechst33258 molecule. In another approach, the Hoechst ligands with a bipyridine unit were shown to assemble on the template DNA mediated by metal coordination (Fig. 1B).[7] It is shown from these studies that assembling motifs with different binding nature may produce unique binding molecules. In this study, we have tested a new system for exploring new DNA binders based on the dynamic combinatorial concept[8] (Fig. 1C). Here we wish to report that an equilibrium containing the Hoechst33258 derivatives (Ht-SH), glutathione (G-SH), and the corresponding homo and hetero disulfides was shifted in the presence of the duplex DNA, and that the ligand with higher binding ability was accumulated.

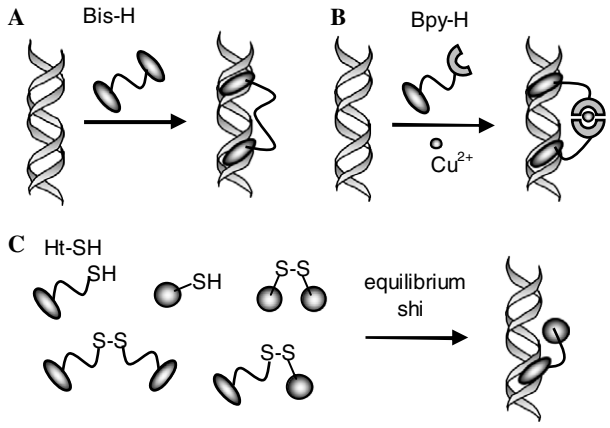

Figure 1. Assembling known DNA-binders creates unique binding properties. (A) Covalently connected bis-Hoechst binds nearby binding sites; (B) non-covalent assembly of Bpy-H is mediated by Cu^{2+} coordination on the DNA template; (C) DNA template shifts equilibrium to produce new DNA binders with relatively higher binding affinity.

Keywords: DNA minor-groove binder; Hoechst33258.
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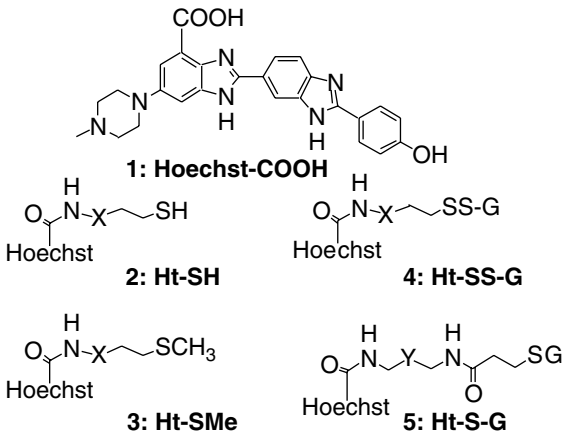

Compds	Abbreviation	Spacer (X or Y)
2, 3, 4	S	X = none
	M	X = $CH_2CH_2OCH_2CH_2O$-
	L	X = $(CH_2CH_2OCH_2CH_2OCH_2CONH)_3$
5	M	Y = CH_2OCH_2
	L	Y = $(CH_2OCH_2)_2CONHCH_2(CH_2OCH_2)_2$

Figure 2. Structures of Hoechst derivatives (**2**–**5**) with different spacer structures. G-SH represents glutathione. Synthesis is described in References and Notes.[9]

The Hoechst33258 derivatives (**2**–**5**) having different lengths of the spacer were synthesized from the carboxylated derivative of Hoechst33258 (**1**)[6] (Fig. 2).[9] The Hoechst-thiol derivatives (**2**) were purified by HPLC (ODS, 0.1% TFA in CH_3CN–H_2O) and tend to form the disulfide. The hetero disulfides (**4**) with glutathione were formed in the equilibrium mixture, which is discussed later. The alkylated compounds (**3** and **5**) were synthesized for the measurements of DNA affinity.

By using mixture containing the sulfide dimer Ht-SS-Ht (**2M-SS-2M**), reduced (G-SH) and oxidized glutathione (G-SS-G), we tested whether an equilibrium is shifted by the addition of the target DNA or not. Ratio of three components of Ht-SS-Ht, Ht-SH and Ht-SS-G was easily measured by HPLC (Fig. 3A). Time-dependent analysis showed that the disulfide (Ht-SS-Ht) was transformed to the monomer (Ht-SH) and the hetero disulfide (Ht-SS-G) immediately after mixing, and that the mixture reached equilibrium after 4 h (Fig. 3B). When DNA (CA14) having a binding motif to the Hoechst33258 unit was added to this equilibrated mixture, the ratio of the hetero disulfide (Ht-SS-G) increased gradually (Fig. 3C). In marked contrast, the addition of CT14 with no binding motif to the Hoechst33258 unit caused slow increase of the homo dimer (Ht-SS-Ht). It is suggested from these results that binding property of the ligands to the DNA is responsible for the different equilibrium shift. Similar experiments were done with other Hoechst33258 ligands with different linker lengths (**2S** and **2L**, Fig. 2). Either the equilibrium mixture containing **2S** or **2L** was not changed by the addition of CT14 (data not shown), whereas the hetero disulfide with glutathione (Ht-SS-G, **4**) increased gradually in the pres-

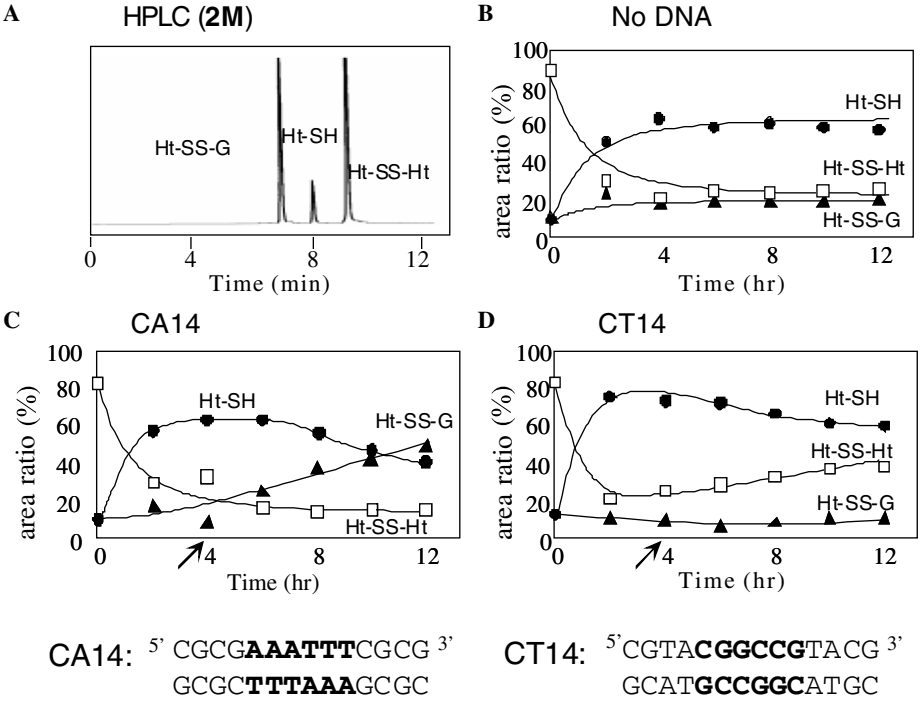

Figure 3. An example of the equilibrium shift with the use of Ht-SH (**2M**) and glutathione (G-SH). (A) HPLC of the mixture; (B) time-dependent change of the components; (C) equilibrium shift after the addition of CA14; (D) equilibrium shift after the addition of CT14. The initial concentrations: Ht-SS-Ht (10 μM); 9.3 mM reduced glutathione (G-SH); oxidized glutathione (G-SS-G) 1.2 mM. Open square: Ht-SS-Ht (**2M-2M**); closed circle: Ht-SH (**2M**); closed triangle: Ht-SS-G (**4M-SG**). DNA (10 μM) was added after 4 h. HPLC conditions: ODS column (4.6 × 250 mm), 1 mL/min, monitored at 370 nm. Solvents: A: 0.5% TFA–H_2O, B: 0.5% TFA–CH_3CN, 20–100% B/20 min, linear gradient.

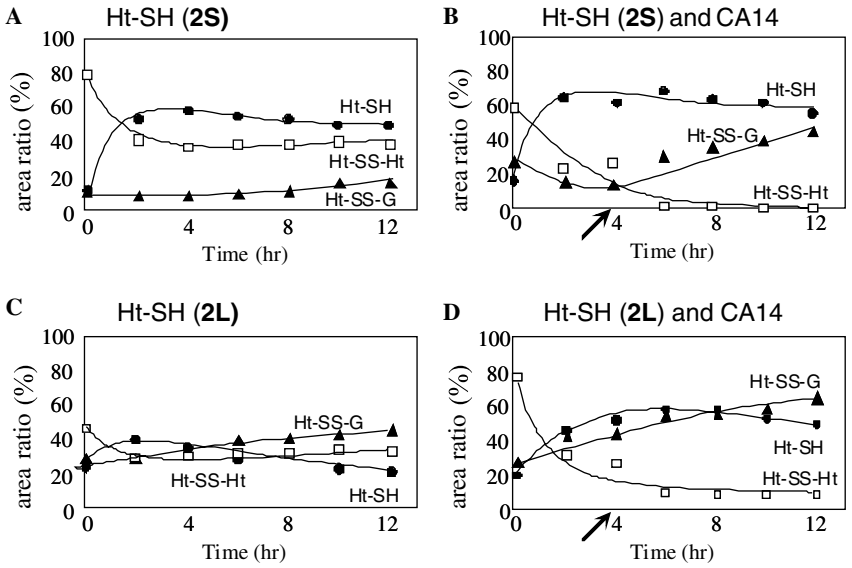

Figure 4. The equilibrium shift with the use of Ht-SH (**2S** and **2L**). (A) The equilibrium mixture with Ht-SH (**2S**); (B) equilibrium shift after the addition of CA14; (C) the equilibrium mixture with Ht-SH (**2L**); (D) equilibrium shift after the addition of CT14. Ht-SS-Ht: open square; Ht-SH: closed circle; Ht-SS-G: triangle. The reaction conditions are the same with the footnote to Figure 3.

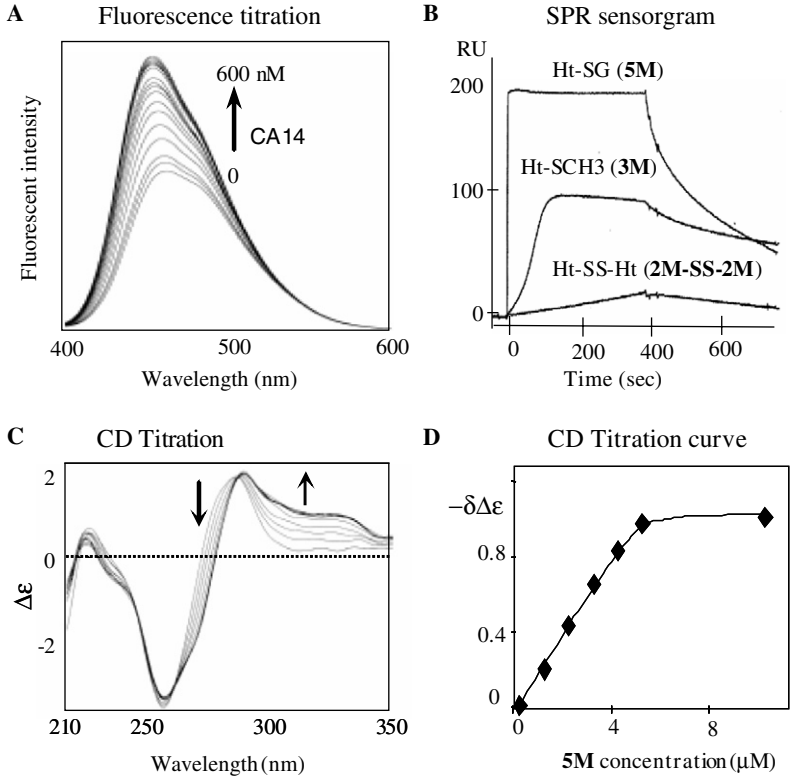

Figure 5. The fluorescence titration (A), the surface plasmon resonance (B), and CD titration (C and D) with the use of **2M**, **3M**, and **5M**. (A) CA14 (0–600 nM) was added to a solution of 100 nM Ht-SG (**5M**) buffered with 50 mM Tris, 150 mM NaCl, and 10 mM $MgCl_2$ at pH 7.4. Fluorescent spectra were measured with excitation at 370 nm. (B) The CA14 biotinylated at the 3′ end was immobilized on the streptavidin–dextran-coated SPR sensor chip, and the surface plasmon resonance was measured by flowing 7.5 μM of each ligand in HBS-N buffer (10 mM Hepes, 150 mM NaCl, and 10 mM $MgCl_2$, pH 7.4). (C) Ht-SG (**5M**) (0–10 μM) was added to CA14 (5 μM) in Hepes buffer (10 mM Hepes, 150 mM NaCl, pH 7.4). (D) Changes of ellipticity at 275 nm are plotted against **5M** concentration.

ence of CA14 (Fig. 4B and D). These results suggested again that the binding property of the ligands affected the equilibrium shift.

In order to confirm that the equilibrium shift is due to difference in DNA binding affinity of each component, we isolated the components; Ht-SH, Ht-SS-Ht, and

Table 1. Equilibrium binding constants of Ht-SS-Ht (**2M-SS-2M**), Ht-SMe (**3M**), and Ht-SG (**5M**) toward the sequence of CA14

Compound	K_s, 10^7 M^{-1}	
	Fluorescence titration[a]	SPR measurement[b]
2M-SS-2M	Could not be determined	Weak
3M	2.6	0.26
5M	3.6	2.2

[a] Values were obtained by the nonlinear least squares data analysis.
[b] Data were analyzed by the software provided by the instrument manufacturer.

Table 2. Equilibrium binding constants of Ht-SS-Ht (**2-SS-2**), Ht-SMe (**3**), and Ht-SG (**5**) toward CA14 obtained by CD titration[a]

Compound	Abbreviation of the spacer (K_s, 10^7 M^{-1})		
	S	*M*	*L*
Ht-SS-Ht (2)	Weak	Weak	0.032
Ht-SCH_3 (3)	10	4.4	0.98
Ht-SG (5)	10[b]	5.7	0.93

[a] Values were obtained by the nonlinear least squares data analysis.
[b] Ht-SS-G (**4S**) was used.

Ht-SS-G. However, it turned out that the thiol and the hetero disulfide resulted in a mixture of the corresponding thiol and disulfide after separation, except the hetero disulfide Ht-SS-G (**4S**). Therefore, the methylated ligand; Ht-SMe (**3**) and the glutathione conjugate; Ht-S-G (**5**), were synthesized (Fig. 2). Equilibrium binding constants of these components were evaluated by fluorescence titration, surface plasmon resonance measurements, and CD titration (Fig. 5).

In the fluorescence titration, fluorescence intensity of the ligand (**3M** or **5M**) was increased by the addition of CA14 having a binding motif for the Hoechst33258 unit, and the binding constants were obtained by the analysis of the titration curve (Table 1). The increase of fluorescence intensity was not observed by the addition of CT14, clearly indicating specific binding to CA14. Although the hetero disulfide Ht-SS-G (**4M**) was expected to have higher binding affinity than the monomer thiol Ht-SH (**2M**) from the equilibrium shift (Fig. 3C), these binding affinities were estimated to be of similar value. Similarly, the homo disulfide (**2M-SS-2M**) was predicted to have weak binding affinity, but fluorescence titration could not be performed properly because of time-dependent decrease of the fluorescence intensity. In the previous study, the ligand connecting two Hoechst33258 units through an ether linker showed similar fluorescence quenching probably because of stacking of two Hoechst33258 units, which might cause weak binding affinity.

We next evaluated binding affinity by surface plasmon resonance by the use of the sensor chip immobilizing CA14 (Fig. 5B). The sensorgram indicated that the binding affinity was in the order of Ht-SG (**5M**) > Ht-SCH_3 (**3M**) > Ht-SS-Ht (**2M-SS-2M**) (Table 1). Although this result seemed to support the data of the equilibrium shift shown in Figure 1C, background signals contained in the SPR sensorgrams due to non-specific binding to the sensor chip decreased reliance on the validity of such conclusion. Therefore, we further applied another method to evaluation of binding affinity.

Previously, we found that CD titration experiments were useful for estimation of DNA binding of the ligand. In this study, it was also found that large CD change was induced by the binding of the Hoechst33258 ligand (Fig. 5C), and that binding constants were calculated by plotting ellipticity against the ligand concentration (Fig. 5D). Binding constants of all ligands are summarized in Table 2.

The weak affinity of the homo disulfide (Ht-SS-Ht, **2-SS-2**) to CA14 (Tables 1 and 2) rationalizes the decrease in the equilibrium shift experiments (Fig. 3C, Fig. 4B and D). The increase of the hetero disulfide (Ht-SS-G, **4**) in the presence of CA14 (Fig. 3C, Fig. 4B and D) might suggest the higher affinity of **4** to CA14. However, this speculation was not clearly proven, because the thiol (**2**) and the hetero disulfide (**4**) or their structural analogs (**3** and**5**) showed similar binding affinity to CA14 (Table 2). A plausible explanation is that experimental errors of the estimated binding constants may be responsible for this discrepancy, and that the equilibrium shift may reflect small differences in affinity. The binding affinities of the derivatives with the spacer of the middle length (**2M**, **3M**, and **5M**) to CT14 were not strong enough to be evaluated by the methods used here. Nonetheless, the equilibrium shift experiment (Fig. 3D) showed the increase of the homo disulfide (**2M-SS-2M**) and the decrease of the thiol (**2M**), probably suggesting the relative order of binding affinities of these components.

Conclusion. In this study, the equilibrium system has been established by the use of the Hoechst33258 derivatives (Ht-SH) having a thiol group with different spacer lengths, reduced and oxidized glutathione. The equilibrium was shifted by the addition of the DNA template depending on the binding affinity of the components. It was shown from the analysis of the components that the hetero disulfide (Hoechst-SS-G) increased by binding with CA14 with an A_3T_3 binding motif for Hoechst33258, and that a different equilibrium shift was induced in the presence of CT14. Thus, a new dynamic combinatorial system for the identification of the new binding ligands has been exhibited. Comparison of the binding affinities of the components suggested that the equilibrium shift might reflect small differences in binding affinity. The equilibrium system composed of the thiol–disulfide interconversion is advantageous in that it can be shifted easily in the presence of the template to identify new binding ligands. A disadvantageous point is that the thiol and the sulfide components suffer interconversion even after separation to make their evaluation troublesome. Further study is now ongoing to overcome the disadvantages of the thiol–disulfide equilibrium system.

Acknowledgments

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9. *Synthesis.* **2S**: (i) **1**, $(ClH_3NCH_2CH_2S)_2$, HOBT, DCC, DMSO, rt; (ii) DTT, H_2O; **2M**: (i) **1**, $BocNHCH_2$–$(CH_2OCH_2)_2CH_2SBz$, HOBT, DCC, DMF, 100 °C; (ii) 0.2 M NaOH; **2L**: (i) three times repetition of the following two procedures with 2-chlorotritylcysteamine resin; (a) $FmocNHCH_2(CH_2OCH_2)_2COOH$, HBT, HBTU, DIPEA, DMF, NMP; (b) 20% piperidine, DMF; (ii) **1**: BOP, HBT, DIPEA, DMF, NMP; (iii) 10% TFA, CH_2Cl_2; **3**: **2**, CH_3I, Et_3N, CH_3OH, **4**: **2**, GSSG, GSH, **5M**: (i) **1**, $H_2NCH_2CH_2OCH_2CH_2NHBoc$, BOP, HBT, DIPEA, DMF; (ii) TFA, CH_2Cl_2; (iii) acrylic acid *N*-hydroxysuccimide ester; (iv) GSH, pH 8, **5L**: (i) two times repetition of the following two procedures with *O*-bis(aminoethyl)ethylene-glycotrityl resin; (a) $FmocNHCH_2$–$(CH_2OCH_2)_2$–COOH, HBT, HBTU, DIPEA, DMF, NMP; (b) 20% piperidine, DMF; (ii) **1**, BOP, HBT, DIPEA, DMF, NMP; (iii) 10% TFA, CH_2Cl_2; (iv) acrylic acid *N*-hydroxysuccimide ester; (v) GSH, pH 8. *Spectral Data:* **2S-SS-2S**: IR (cm^{-1}) 1670, MS (ESI) *m/e* 527.21 $(M+2H^+)/2$, ^1H NMR (CD_3OD–NaOH) δ (ppm), *J* (Hz), 7.97 (2H, s), 7.78 (4H, d, $J = 8.85$), 7.69 (2H, d, $J = 8.54$), 7.45 (2H, d, $J = 2.14$), 7.31 (2H, d, $J = 8.24$), 6.90 (4H, d, $J = 8.56$), 6.83 (2H, d, $J = 2.14$), 4.04 (4H, t, $J = 6.95$), 3.31–3.25 (12H, m), 3.21 (8H, s, br), 2.86 (6H, s). **2M**: IR (cm^{-1}) 1675, MS (ESI) *m/e* 616.21 $(M+H^+)$, ^1H NMR (CD_3OD) δ (ppm), *J* (Hz), 8.48 (1H, s), 8.35 (1H, d, $J = 8.54$), 8.03 (2H, d, $J = 8.85$), 7.87 (1H, d, $J = 8.54$), 7.73 (2H, d, $J = 2.13$), 7.35 (1H, d, $J = 2.14$), 7.07 (2H, d, $J = 8.85$), 3.83–3.74 (8H, m), 3.65–3.64 (4H, m), 3.45 (2H, t, $J = 6, 41$), 3.30–3.12 (8H, m), 3.01 (3H, s), 2.40 (2H, t, $J = 6.41$). **2L**: IR (cm^{-1}) 1668, MS (ESI) *m/e* 482.24 $(M+2H^+)/2$, ^1H NMR (CD_3OD) δ (ppm), *J* (Hz), 8.46 (1H, s), 8.35 (1H, d, $J = 8.55$), 8.03 (2H, d, $J = 7.33$), 7.86 (1H, d, $J = 8.24$), 7.73 (1H, s), 7.34 (1H, s), 7.08 (2H, d, $J = 7.02$), 3.93 (2H, s), 3.90 (2H, s), 3.88 (2H, s), 3.83–3.79 (8H, m), 3.73–3.72 (2H, m), 3.61–3.59 (4H, m), 3.54–3.48 (8H, m), 3.38–3.35 (8H, m), 3.33–3.29 (2H, m), 3.21 (2H, t, $J = 5.04$), 3.01 (3H, s), 2.57 (2H, t, $J = 7.02$).